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beta-Naphthoflavone protects from peritonitis by reducing TNF-alpha-induced endothelial cell activation

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ABSTRACT

β-Naphthoflavone (β-NF), a ligand of the aryl hydrocarbon receptor, has been shown to possess anti-oxidative properties. We investigated the anti-oxidative and anti-inflammatory potential of β-NF in human microvascular endothelial cells treated with tumor necrosis factor-alpha (TNF-α). Pretreatment with β-NF significantly inhibited TNF-α-induced intracellular reactive oxygen species, translocation of $p67^{phox}$, and TNF-α-induced monocyte binding and transmigration. In addition, β-NF significantly inhibited TNF-α-induced ICAM-1and VCAM-1 expression. The mRNA expression levels of the inflammatory cytokines TNF-α and IL-6 were reduced by β-NF, as was the infiltration of white blood cells, in a peritonitis model. The inhibition of adhesion molecules was associated with suppressed nuclear translocation of NF-κB p65 and Akt, and suppressed phosphorylation of ERK1/2 and p38. The translocation of Egr-1, a downstream transcription factor involved in the MEK-ERK signaling pathway, was suppressed by β-NF treatment. Our findings show that β-NF inhibits TNF-α-induced NF-kB and ERK1/2 activation and ROS generation, thereby suppressing the expression of adhesion molecules. This results in reduced adhesion and transmigration of leukocytes *in vitro* and prevents the infiltration of leukocytes in a peritonitis model. Our findings also suggest that β-NF might prevent TNF-α-induced inflammation.

1. Introduction

Vascular inflammatory responses are modulated by complex interactions between circulating leukocytes, tissue-resident leukocytes and the vascular endothelium. Recruitment of leukocytes to endothelium depends on the interactions of the endothelial-cell surface proteins E- and P-selectins with their ligands presented on leukocytes. Vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are most prominently involved in this process [1–3]. Enhanced expression of endothelial cell adhesion molecules and have been proposed to be related to a variety of diseases [4–9]. Exposure to pro-inflammatory molecules such as lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) also leads to a significant increase in the expression of adhesion molecules on the surface of endothelial cells [10–12]. Nuclear factor-κB (NF-κB) may also have a stimulatory effect on adhesion molecules [13,14], while oxidative stress is suggested to be another factor that contributes to the regulation of VCAM-1 [15].

Reactive oxygen species (ROS) have a deleterious effect on vascular functions [16,17]. Superoxide also provokes monocytes to secrete several pro-inflammatory factors such as TNF-α, interleukin-6 (IL-6), interleukin-10 (IL-10) and monocyte chemoat-

Abbreviations: β-NF, β-Naphthoflavone; Egr-1, early growth response gene-1; ERK, extracellular signal-regulated kinase; HUVECs, human umbilical vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

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tractant protein-1 (MCP-1), and all these factors are mediators or modulators of inflammatory reactions in the vascular wall [18]. Recent studies on atherosclerosis suggested that the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex of vascular cells plays a critical role in the production of superoxide in atherosclerotic lesions [19]. It is essential to decrease the production of ROS as they are implicated in the pathogenesis of several diseases.

The compound β-naphthoflavone (β-NF; 5,6-benzoflavone) is a synthetic derivative of a naturally occurring flavonoid compound. It is known to strongly upregulate cytochrome P-450 (CYP) 1A expression via activation of the aryl hydrocarbon receptor (AhR) [20,21], best known for playing important role in regulating the toxicity of xenobiotics such as halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs). Interaction with the HAH 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), results in the AhR-dependent upregulation of several species- and tissue-specific toxic and biological responses [22], including the inhibition of T-cell dependent immune responses [23]. β-NF has also been shown to mitigate dextran sulfate sodium -induced colitis [24].

On the other hand, β-NF can increase the expression of a P450-derived arachidonic acid metabolite, 5,6-epoxyeicosatrienoic acid (EET), which induces hyperpolarization by activation of Ca^{2+}-dependent K^+ channels [25]. It is now known that EETs are endothelium-derived hyperpolarizing factors (EDHFs). A recent report by Node et al. outlined a new autocrine role for EETs in endothelial cells, suggesting they act as anti-inflammatory mediators [26]. Node et al. reported that EETs are predominant inhibitors of CAM expression promoted by TNF-α, IL-1α, and bacterial LPS. Although EETs suppressed the expression of VCAM-1, E-selectin, and ICAM-1, their effects upon VCAM-1 were the most pronounced. However, the effects of β-NF on the vasculum remain unclear.

In this study, we demonstrate that β-NF reduces the expression of adhesion molecules in endothelial cells, and decreases mononuclear cell adhesion by suppressing NF-κB signaling. Our results suggest that β-NF helps to reduce the risk of vascular inflammation by decreasing plasma cytokine release and by directly acting on the vascular endothelium.

2. Materials and methods

2.1. Cell culture

The human microvascular endothelial cells HMEC-1 (ATCC, No CRL-10636) was obtained from American Type Culture Collection (Teddington, UK) and cultured in MCDB131 medium containing endothelial cell growth supplement (Millipore, Billerica, MA, USA) and 15% fetal bovine serum (FBS), as previously described [27]. The human monocytic cell line THP-1 was maintained in RPMI-1640 medium supplemented with 10% FBS.

2.2. Cell viability assays

HMEC-1 cells were grown to confluence in 96-well plates. Upon reaching confluence, medium containing β-NF at various concentrations was added. After a 48-h incubation, the viability of HMEC-1 cells was determined using a WST-1 assay (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions.

2.3. Monocyte-HMEC-1 adhesion assays

HMEC-1 cells were grown to confluence in 24-well culture plates, treated with β-NF for 1 h, and then stimulated with 10 ng/ml TNF-α for 18 h. THP-1 monocytes were suspended in RPMI 1640 containing 0.1% bovine serum albumin (BSA) and labeled with 5 μM Calcein-AM for 30 min at 37 °C; they were then washed twice with Hank's buffered saline solution (HBSS). Fluorescently labeled monocytes (2×10^5 cells/well) were then added and incubated with the β-NF-treated HMEC-1 cells for 30 min at 37 °C. Non-adherent monocytes that had not adhered were removed by gently washing three times with HBBS. Images of adherent THP-1 cells (5 images/well) were obtained using fluorescence microscopy, with the number of bound monocytes counted using AlphaImager 2200 software.

2.4. Transendothelial migration

Migration assays were performed in 24-well 6.5-mm diameter trans-well plates containing polycarbonate membranes with filters (8-μm pore size) (BD Biosciences). Briefly, HMEC-1 cells cultured on trans-well filters were pretreated with increasing concentrations of β-NF for 1 h and stimulated with 10 ng/mL TNF-α for 18 h. THP-1 monocytes (2×10^5 cells/well) were added to the upper chambers of trans-well inserts containing 50 μL of RPMI 1640. After a 4-h incubation at 37 °C/5% CO_2, cells that had migrated to the lower chamber were harvested and counted with a microscope. All experiments were conducted independently at least three times, with the data representing the mean number of THP-1 cells that had migrated.

2.5. Measurement of ROS production

Intracellular ROS production was assessed using 2′,7′-diclorofluorescein diacetate (H2DCFDA; Invitrogen). HMEC-1 cells were treated with β-NF for 1 h and then 10 ng/mL TNF-α for 20 min. HMEC-1 cells were incubated with H2DCFDA (10 μM) for 5 min at 37 °C. Images were obtained using a fluorescence microscope (IX-71, Olympus). Fluorescence intensity was measured using Image J software, averaged, and normalized to the control value. Three independent experiments were performed.

2.6. Transient transfection and luciferase assays

Endothelial cells were grown to 80% confluence and then transiently transfected with plasmids using Lipofectamine (Invitrogen), according to the manufacturer's protocol. Briefly, transfection mixtures contained 0.5 μg of pGL3-4κB-Luc or 0.1 μg of pCMV-β-gal and were mixed with the Lipofectamine reagent before adding to cells for 6 h. Cells were washed and fresh medium was added. After 18 h, cells were treated with β-NF for 1 h and stimulated with TNF-α for 6 h. After cells were lysed, luciferase and β-galactosidase activities were determined using a luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized with respect to β-galactosidase activity and expressed as a percentage of control activity.

2.7. RNA isolation and quantitative polymerase chain reaction (qPCR) assays

HMEC-1 cells were grown to confluence in 6-cm^2 culture plates, treated with β-NF for 1 h, and stimulated with 10 ng/ml TNF-α for 8 h. Total RNA was isolated using Trizol Reagent (Invitrogen), according to the manufacturer's suggested protocol. An aliquot (5 μg) of purified RNA was reverse transcribed into first-strand complementary DNA (cDNA) with a 2720 Thermal Cycler (Applied Biosystems, Grand Island, NY, USA), 200 U/μL M-MLV reverse-transcriptase (Invitrogen) and 0.5 mg/μL oligo(dT)-adapter primers (Invitrogen) in a 20-μL reaction mixture. The qPCR assays for TNF-α, IL-6, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were performed with a Roche LightCycler 480 System (Roche, Indianapolis, IN, USA) and iQ SYBR Green Supermix (Bio-Rad, Hercules,

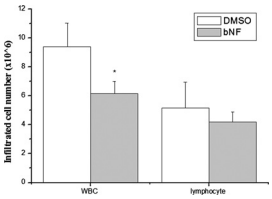

Fig. 1. β-NF alleviates peritonitis inflammation. Cell counts from peritoneal fluid 24 h after intraperitoneal injection of thioglycollate in mice are shown. Values are the mean ± S.E.M. ($n = 10$ for control *vs.* $n = 14$ for the β-NF treatment). $^{*}P < 0.05$ *vs.* DMSO control.

CA, USA). The oligonucleotide primers used were specific for TNF-α (5′-AGG GAC CTC TCT CTA ATC AG-3′ and 5′-TGG GAG TAG ATG AGG TAC AG-3′), IL-6 (5′-GCC GCC CCA CAC AGA CA-3′ and 5′-CCG TCG AGG ATG TAC CGA AT-3′), and GAPDH (5′-ACG GAT TTG GTC GTA TTG GG-3′ and 5′-TGA TTT TGG AGG GAT CTC GC-3′). Thermal cycling conditions involved an initial denaturation step at 95 °C followed by 35 amplification cycles (15 s at 95 °C and 20 s at 60 °C) and subsequent melt curve analysis (72–98 °C). Quantitation of gene expression was conducted relative to GAPDH expression levels.

2.8. Animals and peritonitis model

The animal experiment was used 8- to 10-week-old male BABL/c mice to be animal model. These mice were purchased from the National Laboratory Animal Breeding and Research Center, Taipei. The mice were housed in a temperature-controlled, light-cycled facility and this study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This animal experiment performed by Dr. Shih-Yi Peng was allowed by Tzu Chi University Institutional Animal Care and Use Committee (Permit Number: 101070).

Peritonitis was induced by an intraperitoneal injection of 4% (w/v) thioglycollate in 1 mL of sterile saline (Sigma–Aldrich, USA). Treatment with DMSO or β-NF (20 mg/kg) was performed 1 h before the administration of thioglycollate by intravenous administration. At 24 h after thioglycollate injection, mice were killed by exposure to CO_2, and 5 mL of HBSS was injected into the peritoneal cavity. Cells were obtained by aspirating peritoneal lavage. Differential cell counts were determined using a Hematology Analyzer (KX-21N; Sysmex, USA).

2.9. Statistical analysis

Results are expressed as the means ± S.D. from at least three independent experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA). A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Anti-inflammatory effects of β-NF on a peritonitis model in vivo

We assessed the effects of β-NF on inflammatory cell recruitment using an acute thioglycollate-induced peritonitis model. At 24 h after thioglycollate stimulation, elicited inflammatory cells were detected in the peritoneal cavity. Administration of β-NF 1 h before thioglycollate stimulation significantly reduced total white blood cell infiltration in the peritoneal cavity (Fig. 1). Lymphoycte infiltration was also somewhat reduced as a result of β-NF treatment.

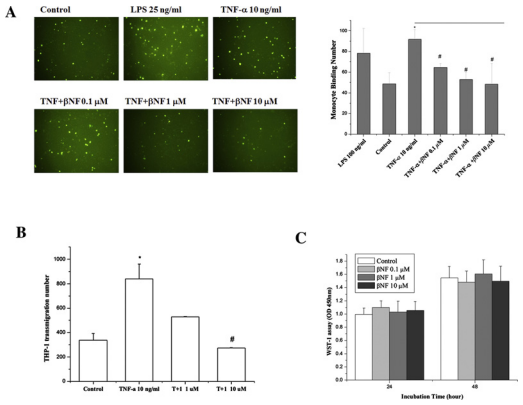

Fig. 2. β-NF inhibits TNF-α-induced adhesion of monocytes to HMEC-1 cells and the transmigration of monocytes. (A) Adhesion of fluorescent THP-1 monocytes was visualized by microscopy and quantified using AlphaImager. Values are the mean ± S.D. $^{*}P < 0.05$ for three independent experiments. (B) THP-1 cells were allowed to transmigrate through the HMEC-1 monolayer towards a TNF-α gradient. Values are the mean ± S.D. of transmigrated monocytes compared with that of controls from three independent experiments. $^{*}P < 0.05$. (C) HMEC-1 cells in 96-well microplate were treated with various concentrations of β-NF. After a 48-h incubation, cell proliferation was evaluated using the colorimetric WST-1 assay. Values are the mean ± S.D. from three independent experiments.

3.2. Monocyte adhesion and transmigration of endothelial cells

To test whether β-NF inhibits cytokine-mediated vascular inflammation, the effects of β-NF on TNF-α-induced monocyte adhesion to HMEC-1 cells were assessed. Normal, confluent HMEC-1 cells bound to THP-1 cells but to a minimal extent. Treatment with TNF-α caused a marked increase in HMEC-1–THP-1 adhesion. Treatment with β-NF decreased the extent of monocyte adhesion to HMEC-1 cells and was concentration dependent (Fig. 2A). β-NF at 0.1 μM significantly reduced the extent of TNF-α-induced monocyte adhesion (0.7 ± 0.11-fold *vs.* TNF-α alone, $n = 3$, $P < 0.05$). The effect of β-NF on monocyte transendothelial migration was studied using trans-well assays. HMEC-1 cells stimulated by TNF-α exhibited increased levels of monocyte adhesion and migration across the endothelium as compared to when TNF-α was absent. In contrast, the ability of THP-1 cells to migrate across HMEC-1 cells was significantly decreased to 90% by the presence of β-NF, in a dose-dependent manner (Fig. 2B). These results show that β-NF can inhibit monocytic migration induced by inflammation. However, the inhibitory effects of β-NF on monocyte adhesion were not due to cytotoxicity, as β-NF had no effect upon cell viability up to 48 h (Fig. 2C).

3.3. Effects of β-NF on TNF-α-induced expression of cell adhesion molecules

We examined whether β-NF could inhibit TNF-α-induced expression of adhesion molecules that mediate leukocyte adhesion to HMEC-1 cells. β-NF pretreatment inhibited TNF-α-induced expressions of ICAM-1 (1.59 ± 0.18-fold relative to control *vs.* 1.32 ± 0.04-fold relative to control for 1 μM β-NF and 1.17 ± 0.07-fold relative to control for 10 μM β-NF, $n = 3$, $P < 0.05$; Fig. 3) and VCAM-1 expression (1.53 ± 0.19-fold relative to control *vs.* 1.29 ± 0.09-fold relative to control for 1 μM β-NF and 1.19 ± 0.06-fold relative to control for 10 μM β-NF, $n = 3$, $P < 0.05$; Fig. 3).

3.4. Anti-oxidative effects of β-NF on ROS production and NADPH oxidase activity

ROS have been shown to activate various transcription factors in cultured endothelial cells and have been implicated as a common second messenger in various pathways leading to NF-κB activation.

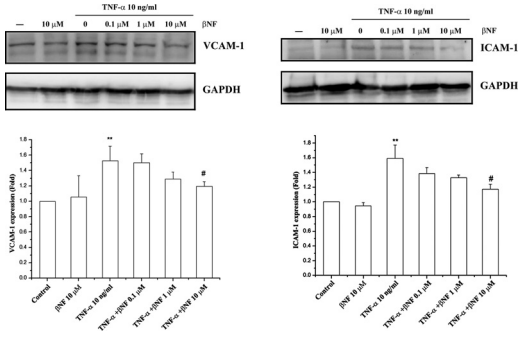

Fig. 3. β-NF inhibits the expression of TNF-α-induced adhesion molecules. Protein expression levels of ICAM-1 and VCAM-1 were measured by western blotting with GAPDH used as a loading control as described in supplementary Materials and Methods. Values are the mean ± S.D. of the protein normalized to GAPDH from three independent experiments. $^{**}P < 0.01$ *vs.* control and $^{\#}P < 0.05$ *vs.* cells stimulated with TNF-α in the absence of β-NF.

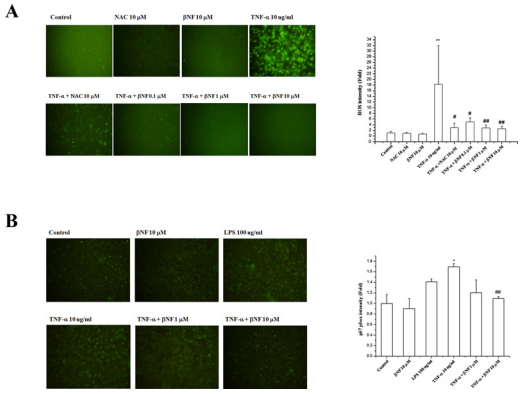

Fig. 4. β-NF inhibits production of TNF-α-induced ROS and $p67^{phox}$ membrane translocation in endothelial cells. (A) ROS production. (B) Immunofluorescence of $p67^{phox}$ membrane translocation as described in supplementary Materials and Methods. Fluorescent intensity was quantified using Image J. Values are the mean ± S.D. of fluorescent intensities *vs.* control from three independent experiments. $^{*}P < 0.05$ *vs.* control and $^{\#\#}P < 0.01$ *vs.* cells stimulated with TNF-α in the absence of β-NF.

To determine whether β-NF could ameliorate the oxidative stress induced by TNF-α, the level of intracellular ROS production was assessed. Treatment with β-NF resulted in a significant decrease of TNF-α-induced ROS production (Fig. 4A). NADPH oxidase in cells comprises membrane-bound ($gp91^{phox}$ and $p22^{phox}$) and cytosolic components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac proteins). NADPH oxidase activation requires the membrane translocation of cytosolic components to associate with membrane-bound components and assemble into an active enzyme. Immunofluorescence staining demonstrated that β-NF pretreatment of HMEC-1 cells resulted in a significant reduction of membrane expression of $p67^{phox}$ after TNF-α stimulation in a dose-dependent manner (Fig. 4B).

3.5. Anti-inflammatory effects of β-NF by inhibiting NF-κB activation

β-NF exerted its anti-inflammatory effects by inhibiting TNF-α-induced oxidative stress and the expression of cell adhesion molecules. The upregulation of cell adhesion molecules and activation of NF-κB can be induced by TNF-α; therefore, we investigated the involvement of NF-κB with respect to the anti-inflammatory effects of β-NF in endothelial cells. To determine whether NF-κB activation by TNF-α is inhibited by treatment with β-NF, western blotting, immunofluorescence staining, and luciferase reporter assays were performed. From the total cell lysate, β-NF was shown

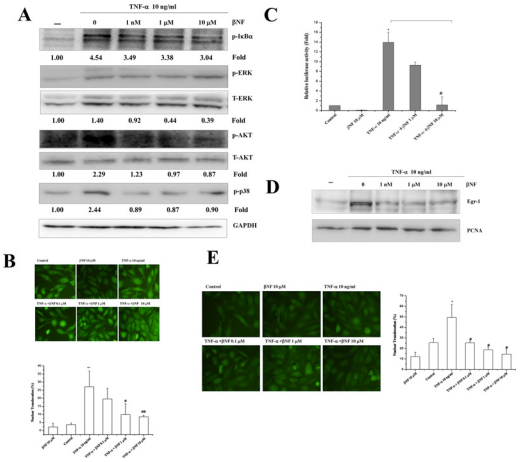

Fig. 5. The anti-inflammatory effects of β-NF are mediated through inhibition of the NF-κB and ERK-Egr-1 pathways. (A) Expression levels of pIκBα, pERK, ERK, pAKT, AKT, pp38, and GAPDH were analyzed by western blotting as described in supplementary Materials and Methods. Results are representative of three independent experiments. (B) HMEC-1 cells were treated with various concentrations of β-NF for 1 h and then stimulated with 10 ng/mL TNF-α for 4 h. NF-κB p65 was detected using rabbit anti-p65 and anti-rabbit FITC-conjugated antibodies (green) as described in supplementary Materials and Methods. Results are representative of three independent experiments. Values are the mean ± S.D. from three independent experiments. $^{*}P < 0.05$ *vs.* control and $^{\#\#}P < 0.01$ *vs.* cells stimulated with TNF-α in the absence of β-NF. (C) Effects of β-NF on TNF-α induced NF-κB luciferase activities were detected. Values are the mean ± S.D. of relative luciferase activities from three independent experiments performed in triplicate. $^{*}P < 0.05$. (D) HMEC-1 cells were treated with various concentrations of β-NF for 1 h, and then stimulated with 10 ng/ml TNF-α for 4 h. Nuclear extracts were harvested, proteins were separated by SDS-PAGE, and Egr-1 expression levels were determined by western blotting. (E) Cells were fixed and stained using an antibody against Egr-1. Translocation of Egr-1 was determined by immunofluorescence. Results shown are from a single experiment, but are representative of three separate experiments. Values are the mean ± S.D. from three independent experiments. $^{*}P < 0.05$ *vs.* control and $^{\#\#}P < 0.01$ *vs.* cells stimulated with TNF-α in the absence of β-NF.

to prevent TNF-α-induced phosphorylation of IκBα in a dose-dependent manner (Fig. 5A). In addition, immunofluorescence analysis revealed that the nuclear translocation of p65, a subunit of NF-κB, and a marker of its activation, was induced by TNF-α treatment. However, pretreatment with β-NF prevented this activation in a concentration-dependent manner (Fig. 5B). Transient transfections were performed using an NF-κB-dependent luciferase reporter plasmid to further examine the effects of β-NF on NF-κB transcriptional activity. Treatment with TNF-α activated NF-κB-luc in endothelial cells, while β-NF efficiently inhibited TNF-α-induced NF-κB luciferase activity (Fig. 5C).

Activation of MAP kinases plays a crucial role during inflammatory responses. For example, AP-1 is a redox-sensitive transcription factor, with the phosphorylation of JNK and ERK occurring prior to AP-1 activation. We detected phosphorylation of JNK, p38, and ERK, and found that phosphorylation levels of ERK and p38 were increased by TNF-α. These increases could be abrogated by β-NF to a certain degree (Fig. 5A). However, β-NF had no influence on phosphorylation of JNK (data not shown). The anti-inflammatory effects of β-NF occurred through the inhibition of NF-κB; however, the involvement of MAP kinases could not be excluded.

Egr-1, a downstream transcription factor involved in the MAPK/ERK signaling pathway, was upregulated by TNF-α treatment (Fig. 5D). We found that pretreatment with β-NF reduced TNF-α-induced Egr-1 expression to 50% (Fig. 5D). To further investigate the effects of β-NF on Egr-1 signaling, we used immunofluorescence to examine the nuclear translocation of Egr-1. Consistent with the expression results, Egr-1 upregulation was inhibited when HMEC-1 cells were treated with β-NF (Fig. 5E).

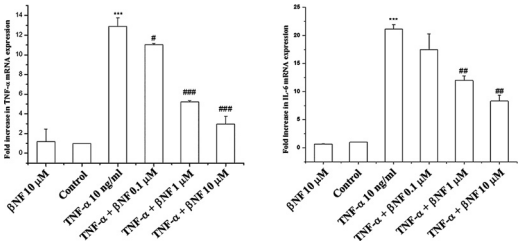

Fig. 6. β-NF adversely affects the expression of TNF-α-induced inflammatory cytokines in HMEC-1 cells. Analysis of TNF-α and IL-6 mRNA levels were determined using qPCR assays as described in supplementary Materials and Methods. GAPDH cDNA was used as an internal control. Values are the mean ± S.D. of mRNA levels relative to those for GAPDH from three independent experiments. $^{*}P < 0.05$ *vs.* control and $^{\#\#}P < 0.01$ *vs.* cells stimulated with TNF-α in the absence of β-NF.

3.6. Inhibitory effects of β-NF on TNF-α-induced cytokine transcription

We used qPCR assays to quantify the effects of β-NF at various concentrations on the expression of TNF-α and IL6 mRNAs (Fig. 6). Compared with control cells, mRNA levels were significantly increased when endothelial cells were cultured with TNF-α. β-NF significantly reduced TNF-α-mediated expression of TNF-α and IL-6 mRNAs. In addition, there was no significant difference in the mRNA expression levels between β-NF-treated and control cells.

4. Discussion

To the best of our knowledge, this study is first to show that, in HMEC-1 cells, β-NF, an AhR activator, inhibits TNF-α-induced expression of cell adhesion molecules. Our findings suggest that β-NF possesses anti-inflammatory properties in endothelial cells. Additionally, we have presented novel evidence to show that the inhibitory effect of β-NF is mediated by ROS, NF-κB and ERK1/2-Egr-1 pathways. AhR is a ligand-dependent transcription factor that mediates environmental and immunotoxic mechanisms [28]. The anti-inflammatory activity of AhR makes it a possible therapeutic target for the treatment of autoimmune inflammation. For example, activation of AhR by TCDD reduces inflammation in experimental autoimmune encephalomyelitis (EAE) [29]. AhR may interact directly with NF-kB and IKKs and suppress proinflammatory responses [24,30]. Selective AhR regulators can also show anti-inflammatory activity, including alleviation of cytokine-mediated acute phase genes, as seen in Huh 7 cells [31]. Recently, Singh et al. showed that AhR activation facilitates epigenetic regulation, thus affecting reciprocal differentiation of Tregs and Th17 cells and alleviating inflammation in murine (C57BL/6) colitis [32]. β-NF has also been known to alleviate DSS-induced colitis [24]. However, the activation of AhR does not always correlate with anti-inflammatory effects. Several reports have shown that β-NF increases oxidative stress [33,34] and enhances hepatocarcinogenesis [33,35,36]. The observation that β-NF can upregulate the cytochrome P-450-dependent monooxygenase system has led to extensive investigation of β-NF as a modifier of chemical carcinogenesis [37,38]. However, Salmonella/microsome assays have shown that β-NF is not mutagenic, with or without metabolic activation induced by Aroclor-1254 [39,40]. Lee et al., also reported that β-NF has no cytotoxic effect on human colon cancer cells [41]. Therefore, β-NF can be considered a putative chemopreventive agent [42]. Controversially, it has been reported that β-NF may have hepatocellular tumor-promoting activity as it increases the surface area and number of preneoplastic foci, positive for placental glutathione S-transferase (GST-P), following N-diethylnitrosamine treatment in rats [35,43]. This increase in tumorigenicity is thought to arise due to stimulation of oxidative stress responses, accompanied by lipid peroxidation and oxidative DNA damage [36,43]. Therefore, the physiological functions of AhR appear to be complex, exhibiting pro-inflammatory or anti-inflammatory characteristics depending on the affected tissue. However, the hepatoma-promoting activity of β-NF reported in previous papers is observed in combination with the carcinogenic agent N-diethylnitrosamine, and treatment with β-NF alone does not examine in the situation. In the present study, we showed that β-NF is beneficial in preventing inflammatory processes in endothelial cells. The reports on the effects of β-NF provide contradictory findings and require further investigation.

Redox regulations in the cell conforms signal transductions that control metabolism, energetics, survival, and cell death. Reactive species are known to act as cell signaling molecules, supporting the double-edged sword of the oxidative stress paradigm in cells [44,45]. Oxidative stress significantly contributes to the pathogenesis of cardiovascular disease. The production of ROS (peroxides and free radicals) is an especially harmful aspect of oxidative stress. Some of the potential sources of ROS in the vasculature are xanthine oxidase, NADPH oxidase, and the mitochondria. Several clinical studies have indicated that enhanced vascular oxidative stress is strongly related to cardiovascular incidents in patients with coronary artery disease [46]. Vascular NADPH oxidases seem to be the most significant source of ROS in inflammatory responses in the vascular wall, causing the development of vascular wall lesions [47,48]. Recent progress in our understanding of atherosclerosis has provided supplementary proof that the NADPH oxidase enzyme complex plays a critical role in the generation of superoxide in atherosclerotic lesions [19]. In an earlier study, we have shown that reduction in the activating subunits of NADPH oxidase, p47 phox and p67 phox, is related to decreased neo-intima formation after carotid ligation in C57BL/6 mice [49]. In this study, we have seen an increase in ROS and NADPH oxidase activity in endothelial cells treated with TNF-α. In cultured cells, addition of NAC, a free radical scavenger, inhibited TNF-α-induced ROS levels. This was an observation that was also made in controls (Fig. 4A). Similarly, treatment with 10 μM β-NF again resulted in reduced ROS levels and NADPH oxidase activity in HMEC-1 cells following TNF-α stimulation (Fig. 4A). Flavonoids are predominant inhibitors of NADPH oxidase activity due to their chemical structure, which comprises a benzene ring with adjacent methoxy–hydroxyl groups [50]. The chemical structure of β-NF is similar to that of flavonoids and is likely to have similar inhibitory effects on NADPH oxidase activity. As shown in our results, β-NF has strong anti-oxidative activity and protects endothelial cells against activation and injury by ROS.

Inflammation is one of the factors that increase the risk of developing cardiovascular disease. The first step in the process of vascular inflammation is the adhesion of monocytes to the endothelium. Subsequently, the monocytes infiltrate the endothelial wall and differentiate into macrophages. This critical step is modulated by interaction between monocytes and the surface molecules of endothelial cells [51,52]. The expression of cell adhesion molecules, such as VCAM-1 and ICAM-1, mediates monocyte and macrophage adhesion as well as other processes that initiate atherogenesis [53]. In our current study, we demonstrated that anti-inflammatory effect of β-NF is related to fewer monocytes adhering to stimulated endothelial cells and inhibition of monocyte transendothelial migration (Fig. 2). This occurs when the expression of adhesion molecules such as ICAM-1 and VCAM-1 is inhibited. As Fig. 3 shows, TNF-α slightly increases ICAM-1 and VCAM-1 expression; however, βNF pretreatment decreases TNF-α induced ICAM-1 and VCAM-1 expression. It was reported that NAC suppresses TNF-α-stimulated upregulation of adhesion molecules by decreasing ROS and NF-κB activity [54,55]. In addition to this,

decline in ROS levels might further prevent cytokine generation in endothelial cells. Treatment with β-NF attenuated production of the pro-inflammatory cytokines TNF-α and IL-6 following TNF-α stimulation (Fig. 6). In our animal model of peritonitis, we observed the inhibition of infiltration of white blood cells and lymphocytes, which confirmed the anti-inflammatory properties of β-NF (Fig. 1). Our results are supported by numerous previous studies that have shown that decreasing intracellular ROS production inhibits the adhesion of monocytes to endothelial cells [56,57].

The redox-sensitive transcription factor NF-κB can be activated by oxidative stress and participates in the production of pro-inflammatory cytokines [58]. The ROS-mediated NF-κB pathway is needed for the transcriptional activation of endothelial ICAM-1and VCAM-1 [59]. NF-κB activation contributes to the development of many chronic diseases, especially atherosclerosis [60]. Phosphorylated IκB-α and subsequently nuclear translocation of NF-κB p65 lead to the activation of specific target genes, including ICAM-1 and VCAM-1. Therefore, we propose that the anti-inflammatory effects of β-NF are due to the inhibition of NF-κB activation. Western blotting analysis and immunofluorescence assays showed that pretreatment with β-NF inhibited TNF-α-stimulated nuclear translocation of NF-κB p65 (Fig. 5). Consistent with this finding, the luciferase reporter assay showed that pretreatment with β-NF attenuated TNF-α-induced NF-κB promoter activity. Oxidative stress enhances NF-κB translocation and the decrease that we observed in NF-κB could be a secondary effect of ROS production suppressed by β-NF [61]. It was discovered that β-NF suppresses the production of TNF-α-induced ROS, indicating its anti-inflammatory function in the ROS-NF-κB pathway in vascular endothelial cells. The effective concentration of β-NF (10 μM) required to suppress the ROS-NF-κB pathway and the effective concentration for reducing the expression of cell adhesion molecules are the same. This finding is supported by the results from a previous *in vitro* study that demonstrated free radical scavenging activity for flavonoids from the fruits of *Trema orientalis* [62]. Perhaps, the protective role of β-NF as a free radical scavenger contributes to its overall anti-inflammatory effects by inhibiting NF-κB activation in vascular endothelial cells. To further confirm whether the observed effects are due to AhR-activation rather than direct interaction with and inhibition of NOX, we performed these experiments using selective AhR antagonist α-Naphthoflavone (α-NF) and AhR siRNA [63]. Effect of AhR siRNA on inhibition of AhR expression was examined and the result is shown in Supplementary data Fig. S 3. According to the result, AhR siRNA partially inhibited AhR expression. Effect of α-NF on TNF-α-induced cytokine responses, ROS and NF-kB translocation and effect of β-NF on TNF-α-induced cytokine responses, ROS and NF-kB translocation in AhR silicing were estimated as Supplementary data Fig. S4–7. The effect of AhR silencing alone on TNF-α-induced various responses was also examined. In these experiments, α-NF had no effect on TNF-α-induced cytokine responses, ROS, and NF-kB translocation. Meanwhile, AhR silencing indeed increased TNF-α-induced cytokine responses, ROS, and NF-kB translocation in comparison with negative control siRNA. β-NF also showed inhibitory ability on TNF-α-induced various responses in both AhR and negative control silencing since the AhR silencing is partially. According to these results, we speculate the observed effects are due to AhR-activation rather than β-NF direct interaction with and inhibition of NADPH oxidase in our system.

Other than the NF-κB/IκB pathway, Akt associated with ERK and p38 MAPK plays a significant role in the signal transduction pathways that modulate the expression of cell adhesion molecules in response to external stimuli such as TNF-α [64,65]. Moreover, PI3K-Akt and p38 MAPK signaling also contribute to NF-κB activation and increased expression of adhesion molecules in response to TNF-α [66,67]. Our results indicate that β-NF greatly suppresses the phosphorylation of Akt, ERK, and p38 MAPK in TNF-α-stimulated endothelial cells (Fig. 5A). These results demonstrate that the anti-inflammatory effects of β-NF are partially due to the inhibition of adhesion molecules through suppression of ERK, p38, MAPK, and PI3K-Akt activation.

An early zinc-finger transcription factor, early growth response gene-1 (Egr-1), contributes to the formation of atherosclerotic lesions [68,69]. Its expression is induced in response to various extracellular stimuli such as growth factors, cytokines, hypoxia, and other harmful stimuli [70,71]. Egr-1 transcription depends on the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) signaling (MAPK/ERK) pathway and serum-response elements of the Egr-1 promoter [72]. Cytokines such as the pro-inflammatory cytokine TNF-α [73], anti-inflammatory cytokine transforming growth factor-β (TGF-β) [74], and basic fibroblast growth factor (bFGF) [75] also have the Egr-1 sequence in their promoter sites [76]. Additionally, Egr-1 transcriptionally modulates ICAM-1, VCAM-1, and coagulation elements such as tissue factors [77]. Several factors rapidly increase the expression of the transcription factor Egr-1 during the progression of atherosclerosis. Once activated, Egr-1 modulates a variety of pro-inflammatory and pro-atherogenic genes in both mice and humans [78]. ERK activation upregulates phosphorylation, which in turn leads to the activation of the transcription factors c-jun, c-fos, and Egr-1 [79–81]. In our study, the level of stimulus-induced Egr-1 was reduced in endothelial cells treated with β-NF (Fig. 5D and E). To our knowledge, this study is the first to show that β-NF inhibits TNF-α-induced expression of VCAM-1 and ICAM-1 through the suppression of the ERK and Egr-1 signaling pathways. However, our results do not exclude other transcription factors and signaling pathways, such as AP-1, SP-1, GATA-2, and IRF-1, that are also involved in the induction of adhesion molecules [82–85]. These findings suggest that the suppressive effects of β-NF on stimulus-induced expression of adhesion molecules and on activation of NF-κB and ERK-Egr-1 and Akt signaling might be due to AhR activation.

5. Conclusion

In summary, our findings indicate that β-NF blocks TNF-α-stimulated NADPH oxidase activity and ROS production, alleviates TNF-α-induced adhesion molecules expressions and monocyte binding, and prevents the infiltration of white blood cells in a peritonitis model. To the best of our knowledge, we have provided the first evidence of the potential benefits of β-NF as a protective agent against vascular inflammation in HMEC-1 cells. Our findings could therefore provide new insights into the pathophysiological mechanisms with regard to the anti-inflammatory properties of β-NF in TNF-α induced endothelial stimulation.

Conflicts of interest

The authors have declared that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.10.001.

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